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SECOND-GENERATION GLYCOSYLATED RGD – COUPLED PEPTIDES ENHANCES TUMOR-SPECIFIC TARGETING, PHARMACOKINETICS AND VASCULAR IMAGING

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ABSTRACT

Previous studies with tracers have shown efficient $\alpha_V\beta_3$-selective tumor uptake, however, these first-generation compounds revealed fast excretion and radioactivity in the liver and intestines. The primary aim of this study was firstly, to improve the pharmacokinetics of these $\alpha_V\beta_3$-selective first generation tracers, and secondly, to allow non-invasive gamma imaging of the $\alpha_V\beta_3$expression. This study describes the synthesis, biological and pharmacokinetic assessment of a second generation glycosylated RGD-coupled peptide derivative, in order to decrease hepatobiliary uptake, and improve its pharmacokinetics. The precursor cyclo(-Arg-Gly-Asp-d-Tyr-(SAA)-GP1 was generated through coupling 3-acetamido-2,6-anhydro-4,5,7-tri-*O*-benzyl-3-deoxy-β-d-glycero-d-gulo-heptonic acid (SAA(Bn_3)) with cyclo(-Arg(Mtr)-Gly-Asp-d-Tyr(tBu)-Lys-)**,** after which the protection groups were removed. The Iodo-Gen method was used for Iodine labeling, *in vitro* binding assays were performed using purified immobilized $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins, while nude mice bearing either xenotransplanted melanomas or osteosarcomas were used for the *in vivo* experiments. The glycosylated peptide 3-iodo-Tyr-cyclo(-Arg-Gly-Asp-d-Tyr-(SAA)-GP2 showed high affinity and selectivity for $\alpha_V\beta_3$*in vitro* as well as specificity to $\alpha_V\beta_3$-expressing tumors *in vivo.* Comparison of the pharmacokinetics of [^{125}I]GP2 and [^{125}I]-3-iodo-Tyr^4-cyclo(-Arg-Gly-Asp-d-Tyr-Val)-P2 revealed an increased activity concentration in the blood (e.g., 3.49 ± 0.45 percentage injected dose [%ID]/g vs. 1.62 ± 0.44 %ID/g at 10 min post-injection) and a markedly reduced accumulation in the liver (e.g., 2.49 ± 0.34 %ID/g vs. 24.76 ± 2.65 %ID/g at 10 min postinjection) for [^{125}I]GP2. Furthermore, increased accumulation in the tumor was found with the GP2 at 240 min postinjection, which remained almost constant between one and four hours post-injection. This study demonstrates that the coupling of a sugar moiety enhances the pharmakokinetic characteristics of a hydrophobic tracer that allows for both non-invasive visualization of $\alpha_V\beta_3$-expressing. Furthermore tumors and potential monitoring with $\alpha_V\beta_3$ antagonists. Furthermore, the glycosylated RGD-peptide improves the tumor-to-blood ratio, making it a lead structure for optimizing the quantification of $\alpha_V\beta_3$ expression towards effective therapeutics.

KEYWORDS: RGD, Tumor Targeting, Pharmacokinetics, Vascular Imaging

INTRODUCTION

The integrin $\alpha_V\beta_3$,has been well characterized for its involvement in angiogenesis and tumor invasiveness, Felding-Habermann B & Mueller BM {1992}.The integrin is expressed on various malignant tumors as well as on endothelial cells during neovascularization (Felding-Habermann B & Mueller BM {1992}; Friedlander M, Brooks P.C. et al., {1995}. In tumor models, inhibition of blood vessel formation using $\alpha_V\beta_3$ antagonists blocked both tumor-associated angiogenesis and tumor regression (Brooks P.C., Montgomery A.M et al {1994}, Brooks P.C. & Stromblad S. {1995}. Protocols to help visualize and quantify the in vivo expression of $\alpha_V\beta_3$ expression seems to be imperative for the clinical application of $\alpha_V\beta_3$ antagonists in cancer patient, Brower V. {1999}.

Radiolabeled cyclic RGD-peptides with high affinity for the $\alpha_V\beta_3$ integrin, Haubner R. & Wester H.J. {1999}.has been reported. These peptides showed receptor-specific accumulation in different mouse models. However, they also showed enormous hepatobiliary excretion. The high activity concentration in the liver is a limiting factor on tracers for tumor imaging. The aim of this study was therefore to improve the pharmacokinetics of the $\alpha_V\beta_3$-specific tracers to allow noninvasive imaging of $\alpha_V\beta_3$ expression with gamma camera imaging techniques. For this investigation, glycosylation of a modified derivative of the peptides with a sugar amino acid was found to signifiantly reduce lipophilicity and hepatic uptake.

METHODS

Peptide Synthesis. Synthesis of Fmoc-3-iodo-d-Tyr-OH has already been described elsewhere Haubner R. & Wester H.J. {1999}.. 1-Hydroxybenzotriazol (HOBt), O-(1H-benzo-triazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate, and diphenyl phosphorazidate were purchased from Alexis (Grünberg, Germany). Sodium iodide-125 and sodium iodide-123 were purchased from Amersham (Buckinghamshire, UK). All other organic reagents were purchased from Aldrich or Fluka (St. Louis, MO). Mass spectra were recorded on the liquid-chromatography mass-spectrometry system LCQ from Finnigan (Bremen, Germany) using the Hewlett-Packard series 1100 high-performance liquid chromatography system. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AMX 500 (Karlsruhe, Germany) at 300 K. Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed on Sykam equipment (Gilching, Germany) using columns with YMC-Pack ODS-A (5 µm, 250 × 4 mm) (YMC Co., Ltd., Kyoto, Japan). For radioactivity measurements, the ultraviolet detector was connected to a well scintillation NaI(Tl) detector from EG & G (Munich, Germany).

Preparation of the Sugar Amino Acids. Synthesis of The benyzl-protected sugar amino acid 3-acetamido-2,6-anhydro-4,5,7-tri-O-benzyl-3-deoxy-β-d-glycero-d-gulo-heptonic acid (SAA(Bn_3)) was synthesized according to Hoffmann et al. (13). In the synthesis of 3-Acetamido-2,6-Anhydro-3-Deoxy-β-d-Glycero-d-Gulo-Heptonic Acid, SAA(Bn_3) (0.2 mmol) was dissolved in a mixture of MeOH)/water (3:3:1). After addition of palladium oxide and 200 µL acetic acid (HOAc), and stirred, the suspension was filtered, followed by in vacuo solvent removal. The crude product was freeze-dried and the resulting sugar amino acid was used without further purification.

Synthesis of Cyclic Pentapeptides. TheLoading of the TCP-resin, peptide synthesis, and the cyclization thereafter were performed as previously described Haubner R. & Wester H.J. {1999}.. Protection of the side chainswas achieved with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for benzyloxycarbonyl, Haubner R. & Wester H.J. {1999}. and tBu for aspartic acid and tyrosine. The sensitivity of iodo-tyrosine to reducing conditions by hydrogen or palladium,

neccessitated the deployment of two different synthesis for the reference peptide as well as the labeling precursor. This resulted in cyclo(-Arg(Pbf)-Gly-Asp(OtBu)-d-3-iodo-Tyr-Lys(Dde)-) (peptide precursor for the reference compound) andcyclo(-Arg(Mtr)-Gly-Asp(OtBu)-d-Tyr(tBu)-Lys(Z)-) (peptide precursor for labeling).

Removal of Protection Groups. Selective Removal of Z-Protection Group and the Dde Protection Group were done as previously described **(6)**. Removal of Side Chain Protection Groups of Peptides as ell as the benzyl groups have already been described **(6).** The crude cyclic peptides and glycopeptides were purified by RP-HPLC. Analytical data, including ^{1}H- and ^{13}C-chemical shift data for GP1, are given in Tables 1 and 2.

Radioiodination. The cyclopeptides P1 and GP1 were labeled with ^{125}I or ^{123}I (the last only for GP1) using the IODO-GEN method, mixed with 100 μL of PBS (pH 7.5). The individual solutions were added to 250 μg IODO-GEN and combined with 10-15 μL no-carrier-added (NCA) [^{125}I]NaI (30–80 MBq) or 25 μL carrier-added (CA) [^{123}I]NaI (185 MBq). Purification was performed using RP-HPLC and eluted with 2 mL methanol. The resultant residue was dissolved with PBS to obtain solutions with an activity concentration of 375 kBq/100 μL for use in animal experiments. The overall radiochemical yield after RP-HPLC was ~60%.

Biological Assay. Protein purification and integrin-binding assays have been described previouslyHaubner R. & Wester H.J. {1999}.. The cyclic peptide inhibition was assayed by quantifying their effect on the interactions between immobilized integrin and the biotinylated vitronectin or fibrinogen ligands. As controls, the use of recombinant human $\alpha_V\beta_3$ and recombinant soluble human $\alpha_V\beta_5$ produced similarlyyielded identical results to the placental integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Since the integrin preparations altered with time, the linear peptide and the $\alpha_V\beta_3$-selective cyclic peptide were used as internal controls to allow for interassay standardization.

Tumor Xenografts. Biodistribution of [^{125}I]P2 and [^{125}I]GP2 was evaluated in murine osteosarcoma and a xenotransplanted human melanoma model which have high expression of the $\alpha_V\beta_3$ integrin (Haubner R. & Wester H.J. {1999}; Cheresh D.A. & Spiro R.C. { 1987}. Murine osteosarcomas were transplanted into BALB/c mice. Tumor were injected close to the femur into the quadriceps. Mice weighing between 20-25 g, and having tumor weights of~500 mg were the source of further investigations. Human M21 melanoma cells **(7)** were cultured using RPMI 1640 medium supplemented with 10% fetal calf serum. Tumor xenografts were obtained by subcutaneous injection of 5×10^6 cells and mice weighing between 20-25 g bearing tumors beteen 300–400 mg were used for biodistribution.

Biodistribution Studies. Nude mice bearinghuman melanoma M21 tumor xenografts and osteosarcome-bearing BALB/c mice were injected intravenously with 200–300 kBq [^{125}I]P2, [^{125}I]P4, or [^{125}I]GP2. Tail vein injections were done after ether anesthesia. The animals were killed and dissected at different times post-injection of the ^{125}I-labeled peptides. Blood, plasma, kidney, heart, liver, muscle, brain, spleen, lung, thyroid, intestine, and tumors were removed and weighed. Radioactivity in the various tissues was quantified with a gamma counter. Results are expressed as the percentage injected dose per gram of tissue (%ID/g).

Blocking and Pretreatment Studies. Blocking of the $\alpha_V\beta_3$ integrin was performed as previously described by injecting either 3 mg/kg or 6 mg/kg before injection of of [^{125}I]P2 and [^{125}I]GP2) respectively(Aumailley M., Gurrath M. et al., {1991},for 15 min. 200–300 kBq of the radioactive compound per 100 μL of PBS (pH 7.4) was subsequently injected. Animals were killed and dissected 60 min after injection of the ^{125}I-labeled peptides. Further processing was performed as described above.

Gamma-Camera Imaging. Osteosarcoma-bearing BALB/c mice were injected intravenously with 5.6 MBq [^{123}I]GP2. and killed 4 h after injection. Gamma camera images (Siemens Multispect 3; Hoffman Estates, IL) were then obtained with acquisition time of 20 min/image.

RESULTS

In Vitro Binding Assay The binding of vitronectin and fibrinogen to the immobilized $\alpha_{IIb}\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_3$ receptors was evaluated in the presence of cyclic pentapeptides, and compared to that of the linear low-affinity peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys and the $\alpha_V\beta_3$-selective cyclic (-Arg-Gly-Asp-d-Phe-Val-) peptide as internal standards. The inhibitory peptides suppressed the ligand-receptor binding. Table 3 shows the inhibitory capacities of P2 being in the same range as those for cyclo (-Arg-Gly-Asp-d-Phe-Val-). P4, GP1 and GP2 revealed 2-4 higher IC_{50} values for all integrins. The biological activities are 100–150 times higher for the $\alpha_V\beta_3$ integrin than for $\alpha_V\beta_5$ or $\alpha_{IIb}\beta_3$. The negative control peptide cyclo(-Arg-d-Ala-Asp-Tyr-Val-) P5 showed no activity for the experimental range used for $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$.

Biodistribution Studies. In the melanoma model, initial liver uptake of [^{125}I]GP2 was 2.6 ± 0.2 %ID/g and 22 ± 2.8 %ID/g for [^{125}I]P2. Peptide structures are shown in Figure 1. In contrast differences in renal tracer uptake were relatively small for the time points studied. The blood clearance of [^{125}I]P2 was three times more rapid than that for [^{125}I]GP2. Tumor uptake of [^{125}I]GP2 was higher than that for [^{125}I]P2 at all time points. At 240 min after tracer injection, the tumor-to-blood ratio (T/B) was slightly higher for [^{125}I]GP2 than for [^{125}I]P2 (8.9 vs. 6.8, respectively) while the thyroid activity concentration was 0.3 ± 0.1 %ID/g for [^{125}I]P2 and 11.8 ± 6.5 %ID/g for [^{125}I]GP2. With the exception of the intestines, all other organs showed marginal uptakes of [^{125}I]P2 and [^{125}I]GP2. Activity concentration in muscle, heart, and lung was about twofold higher for [^{125}I]GP2 than for [^{125}I]P2. The tumor-to-muscle ratio (T/M) at 4 hrs postinjection was 4.2 for [^{125}I]P2 and 7.1 for [^{125}I]GP2.

In the osteosarcoma model, tumor uptake of [^{125}I]GP2 was 3.5-fold higher than that of [^{125}I]P2 (3.2 ± 0.3 %ID/g vs. 0.9 ± 0.3 %ID/g). At this time point, the Tumor to Blood ratio was 16.0 for [^{125}I]GP2, and only 7.7 for [^{125}I]P2. The radioactivity concentration for both tracers in the thyroid was considerably higher than that for the melanoma (nude mouse) model ([^{125}I]P2: 30 ± 11 %ID/g; [^{125}I]GP2: 175 ± 34 %ID/g). For all other organ systems, trends in tracer uptake of [^{125}I]P2 and [^{125}I]GP2 were similar to those in the melanoma model.

The summaries of the tissue distribution of [^{125}I]P2 and [^{125}I]GP2 areshown in Table 4 and Figure 2. There is a variance in the thyroid uptake, which could have resulted fromthe small amounts of free iodine in the different preparations used for the biodistribution studies. However, the low thyroid uptake due to the low thyroid mass was not expected to influence the overall quality of the gamma image. This was also confirmed by gamma-camera imaging.

Blocking and Pretreatment Studies Figure 3 shows the results of the pretreatment studies with [^{125}I]P2 and [^{125}I]GP2 using the melanoma/mouse model. Blocking with 3 mg of the cyclic peptide -Arg-Gly-Asp-d-Phe-Val for 10 min before [^{125}I]P2 and with 6 mg of cyclo(-Arg-Gly-Asp-d-Phe-Val-) for 10 min before [^{125}I]GP2 injection, respectively, reduced the activity accumulation in the tumor to ~0.5 %ID/g at 60 min postinjection for both tracers.

Gamma-Camera Imaging Figure 4 shows the image at 4 h postinjection of [^{123}I]GP2. It prominently shows a contrasting tumor on the left flank of the mouse with just an insignificant and marginal signal in the background. High-activity concentrations also were found in the intestine and in the unblocked thyroid.

CONCLUSIONS

Several studies indicate that sugar moieties can be used to improve peptide solubility under physiological conditions, (Michael K., Wittman V. et al., {1996}), bioavailability, (Kihlberg J, Ahman J., et al., {1995}, Alpert R., Marbach P., {1993}), the crossing the blood-brain barrier, and resistance against proteases (Marastoni M., Spisani S., {1994}).Structurally, the presence of the C—C bond at the anomeric center of the amino acid results in C-glycosylated peptides that are very stable against either denaturing or metabolic degradation. In this report, we have described the introduction of a sugar amino acid to improve the pharmacokinetics of radiolabeled RGD-containing pentapeptides. We further demonstrate that the optimized glycosylated tracer is amenable to structural modifications and allows for high-quality gamma imaging of $\alpha_V\beta_3$-expressing tumors in nude mice. These data suggest the potential use of this tracer in the planning of antiangiogenic therapies.

The glycosylated second-generation tracer was primarily derived from the $\alpha_V\beta_3$-antagonist cyclo(-Arg-Gly-Asp-d-Phe-Val-) (Gurrath m., Muller G., et al., {1992})which demonstrated that substitution of the amino acid adjacent to the arginine residue had no influence on selectivity and affinity for $\alpha_V\beta_3$. Thus, valine of P2 was substituted with lysine, allowing the conjugation of the side chain amino group with the carboxy group of the sugar amino acid (Figure 1). Comparison of the ^{1}H- and ^{13}C-chemical-shift data of GP1 (Table 2) with varying cyclic pentapeptides had no effect on the bent conformation of the RGD-side, which is important for high $\alpha_V\beta_3$ affinity and selectivity.

In the study by Friedlander et al. {1995}.**,**cyclic peptide -Arg-Gly-Asp-d-Phe-Val-, generatedpreviously (Aumailley et al., {1991}; Pfaff et al., {1994), was inhibitedwithout recourse to the mode of stimulation. Thus, the described tracers should allow monitoring of tumor-induced angiogenesis that is independent of the type of α_V integrin. However, in our in vitro assay, neither the cyclic -Arg-Gly-Asp-d-Phe-Val-and its derivatives described here showed only nominal affinites for $\alpha_V\beta_5$. This different behavior both in vitro and in vivo will be subject to future experiments. Competition experiments with cyclo(-Arg-Gly-Asp-d-Phe-Val-) demonstrated receptor-specific accumulation of [^{125}I]GP2 in vivo (Figure 2). Pretreatment of the mice with the cold peptide clearly reduced the activity accumulation in the tumor. This finding has been confirmed by other investigatorsusing an iodated nonspecific control. These results indicate that [I]GP2 could potentially be used to block the $\alpha_v\beta_3$ integrin during therapy with unlabeled antagonists such as SM256 (Kerr J.S., Wexler R.S., et al., {1999}and SC68448 (Carron C.P., Meyer D.M., {1998}).

Comparison of the biodistribution data of [^{125}I]P2 with [^{125}I]GP2 (Figure 2) showed that [^{125}I]P2 preferred the hepatobiliary elimination pathway and [^{125}I]GP2 the renal elimination pathway. This result indicates that the more hydrophobic [^{125}I]P2 and [^{125}I]P4 were eliminated rapidly from the circulatory system, whereas the hydrophilic [^{125}I]GP2 (as calculated in Table 1) remained slightly longer in the circulatory system, resulting in an increase in tracer uptake in the tumor bearing the glycosylated tracer. The significantly slower blood clearance of [^{125}I]GP2 may be attributable to the higher activity concentration in muscle, lung, and myocardium. Nevertheless, introduction of a hydrophilic group improved the pharmacokinetics and tracer in the $\alpha_V\beta_3$-expressing tumors. In addition, the tracer uptake in both tumor models seemed fairly constant between 1 and 4 hrs postinjection.

The gamma image of an osteosarcoma-bearing mouse at 4 hrs postinjection of [^{123}I]GP2 confirmed the significant tumor-to-organ ratios. Thyroid uptake was slightly higher than that for [^{125}I]P2, but it still reflected a low accumulation in

the entire thyroid (0.45 %ID for a 2mg thyroid weight). This indicated that deiodination in vivo still renders [*I]GP2 stable.

Radiolabeled RGD-peptides may be used to document $\alpha_V\beta_3$ expression in tumors before administration of $\alpha_V\beta_3$–specific antagonists. While this allows selection of patients entering a clinical trial, the optimum dosage for treatment with these drugs may also be determined. Further, quantifying the uptake of RGD-peptides may be an empirical measure for angiogenic activity, because $\alpha_V\beta_3$ is predominantly expressed on activated but not on quiescent endothelial cells**(**Gasparini G. {1984}**),** however, empirical studies will be required to determine whether the signal obtained in vivo is angiogenic activity-specific. This is coming against the backdrop of the fact that, several common pathways inter-connect the various processes,culminating in the formation of newly formed vessels during tumor invasiveness and tumor-induced angiogenesis. Several in vitro studies have implicated $\alpha_V\beta_3$ in both processes **(**Carron C.P., Meyer D.M., {1998}, Eliceiri B.P., Cheresh D.A., {1999}).Furthermore, clinical studies have supported the experimental data by showing a positive correlation between $\alpha_V\beta_3$ expression and patient survival (Gasparini G., Brooks P.C., et al., {1998}).Finally, imaging of $\alpha_V\beta_3$ expression plus densitometric assessmentwould come in very handy in asessing the aggression and pervasiveness of malignant tumors in patients.

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